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# Expression of calcitonin gene-related peptide, adrenomedullin, and receptor modifying proteins in human adipose tissue and alteration in their expression with menopause status

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## Abstract

**Objective:** Calcitonin gene-related peptide (CGRP) is a vasoactive, proinflammatory neuropeptide implicated in the pathogenesis of cardiovascular disease. Elevated CGRP levels during hot flushes and pregnancy suggest that reproductive hormones may influence CGRP secretion. CGRP and the related protein adrenomedullin (ADM) may function through adipose tissue-mediated effects, since adipose tissue is an important site of cytokine production and the main site for estrogen production after menopause. This study examined mRNA and protein expression of CGRP, ADM, and the receptor activity-modifying proteins and the effects of menopausal status in human adipose tissue.

**Design:** Protein/mRNA levels were determined in adipose tissue biopsy samples collected from premenopausal (n = 22: follicle-stimulating hormone, <20 IU/L; estradiol [mean ± SE], 434.5 ± 87.81 pmol/L) and postmenopausal (n = 25: follicle-stimulating hormone, >20 IU/L; estradiol, 43.4 ± 6.95 pmol/L) women.

**Results:** Our studies determined that CGRP, ADM, and receptor activity-modifying proteins were expressed in abdominal fat, adipocytes, and preadipocytes. CGRP and ADM mRNA levels were increased in abdominal subcutaneous fat in postmenopausal women compared with premenopausal women (βCGRP: premenopause Δ cycle threshold [Ct], 31.07 ± 0.28 vs postmenopause ΔCt, 30.35 ± 0.17, *P* = 0.035; ADM: premenopause Sc ΔCt, 12.41 ± 0.2 vs postmenopause Sc ΔCt, 11.55 ± 0.14, *P* < 0.001) with CGRP differentially expressed in subcutaneous and omental depots. CGRP protein expression was higher in postmenopausal women (*P* < 0.05) in both fat depots.

**Conclusions:** Our findings suggest that adipose tissue represents an important site for CGRP and ADM production and that menopause status alters their expression in abdominal fat. This offers a potential mechanism to explain the role of CGRP in menopausal vasomotor symptoms and the increased risk of cardiovascular disease in postmenopausal women.

**Key Words:** Calcitonin gene-related peptide – Adrenomedullin receptor-modifying proteins – Menopause – Adipose tissue.

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The menopausal transition in women is associated with weight gain, increased visceral fat content, and central adiposity.<sup>1</sup> Although recent studies have shown higher body mass index (BMI) to be associated with increased

severity of hot flushes in postmenopausal women,<sup>2-7</sup> the mechanisms through which adipose tissue (AT) influences the severity of these symptoms is not yet known. Calcitonin gene-related peptide (CGRP), a vasoactive peptide, may have a role in the onset of hot flushes. Elevated CGRP serum levels have been observed during hot flushes in postmenopausal women and in men after castration,<sup>8-10</sup> linking acute release of CGRP with the onset of vasomotor symptoms. Animal and human studies have demonstrated that secretion and activity of CGRP and adrenomedullin (ADM) are influenced by the sex hormones. Increased plasma levels of CGRP have been noted during pregnancy, although its hypotensive effects were potentiated in the presence of the sex hormones.<sup>11,12</sup> In postmenopausal women, hormone therapy (HT) was associated with increased plasma CGRP levels.<sup>13</sup> Similarly, circulatory ADM levels are influenced by the phase of menstrual

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cycle, and plasma levels are increased during normal pregnancy.<sup>14,15</sup>

CGRP is a 37-amino-acid neuropeptide that exists in two forms,  $\alpha$  and beta  $\beta$ , that differ by three amino acids in humans.<sup>16</sup> It is widely distributed throughout the body, with  $\alpha$ CGRP being the predominant form in the central and peripheral nervous system, although  $\beta$ CGRP is present in the enteric system and pituitary gland.<sup>17</sup> However, the importance of their differential distribution and regulation in vivo is not yet clear. Both isoforms have potent vasodilatory capacity, although studies have suggested they may have different signaling and regulatory mechanisms at the receptor level.<sup>18,19</sup>

ADM, a 52-amino-acid peptide, is another member of the calcitonin family and is produced by a variety of cells, including vascular endothelial and smooth muscle cells.<sup>20</sup> Although ADM is mainly active at the microvasculature level, it is also present in the circulation in considerable amounts.<sup>21,22</sup> CGRP is secreted in the tissues by sensory nerves and rapidly metabolized by tissue endopeptidases; therefore, the small amount detectable in the circulation is thought to be a spillover from the perivascular sensory nerve endings.<sup>23,24</sup> These studies suggest that local paracrine effects may be more important than its systemic influence. Both CGRP and ADM bind to a common receptor molecule called calcitonin receptor-like receptor (CRLR); however, for the receptor to be functional, an additional transmembrane protein called receptor activity-modifying protein (RAMP) is required. Three RAMPs (RAMP1, RAMP2, RAMP3) determine the specificity of the receptor to either CGRP or ADM because in the presence of RAMP1, the CRLR acts primarily as a CGRP receptor, whereas in the presence of RAMP2 and RAMP3, it acts as an ADM receptor (ADM<sub>1</sub> and ADM<sub>2</sub>). Alteration in RAMP expression provides another potential mechanism through which cells may change their responsiveness to CGRP and ADM.<sup>25</sup>

We hypothesized that the local activity of AT-derived CGRP may influence the severity of hot flashes by regulating cutaneous vasodilatation. Therefore, the aim of the study was to determine whether CGRP was expressed in human AT and whether its expression was altered by menopausal status. Because many AT-derived cytokines are depot specific, we examined the expression of CGRP in paired samples of subcutaneous (Sc) and omental (Om) AT. In addition, we explored the possibility that expression of ADM and the RAMPs in human AT may be influenced by menopause status.

## METHODS

### Participants

For these studies, fasting blood samples ( $n = 31$ : premenopausal, 22; postmenopausal, 9) and paired Sc and Om AT samples were obtained from premenopausal and postmenopausal women ( $n = 30$ : premenopausal, 22; postmenopausal, 8) undergoing elective abdominal surgery for benign conditions. Additionally, 17 unpaired abdominal Sc

(AbSc) AT samples were also collected from postmenopausal women. Postmenopausal status was defined as amenorrhea for more than 12 months along with serum follicle-stimulating hormone (FSH) levels greater than 20 IU/L. In total, 77 ex vivo AT samples were analyzed, comprising premenopausal AbSc ( $n = 22$ ), premenopausal abdominal Om (AbOm) ( $n = 22$ ), postmenopausal AbSc ( $n = 25$ ), and postmenopausal AbOm ( $n = 8$ ). Women with any medical condition (ie, hypertension, cardiovascular disease, thyroid disorders, renal disorders, diabetes, or chronic pain conditions) were excluded from the study. None of the women were on endocrine therapy (ie, HT, tamoxifen, steroids, or antiglycemic agents) or any medication that might have influenced CGRP secretion. All samples were collected in accordance with the research protocol approved by the Solihull Local Research Ethics Committee.

### Collection of clinical data

Anthropometric data were collected to calculate BMI and waist-hip ratio. Systolic and diastolic blood pressures were recorded before sample collection. The date of last menstrual period, cycle length, and regularity were recorded to determine the menopause status in postmenopausal women (last menstrual period 1-36 y) and phase of menstrual cycle in premenopausal women. Women who were within the last 2 weeks of their cycle were classified as being in the luteal phase ( $n = 11$ ), and women who were in the earlier part of their cycle were classified as being in the follicular phase ( $n = 6$ ).

### Collection, storage and analysis of samples

Fasting blood samples (minimum 6 h) were collected and analyzed for serum glucose, insulin, estradiol, and FSH. After collection, the serum and AT samples were immediately stored at  $-80^{\circ}\text{C}$  until they were required for analysis. Estradiol, FSH, and luteinizing hormone levels were determined using electrochemiluminescence immunoassays (Roche Diagnostics) (estradiol reference range, 18.4-15,781 pmol/L; coefficient of variation [CV]: intrabatch, 5.7%; interbatch, 6.2%; FSH reference range, 0.1-200 IU/L; CV intrabatch, 2.8%; interbatch, 4.5%; luteinizing hormone reference range, 0.1-200 mIU/mL; CV intrabatch, 1.8%; interbatch, 5.2%). The samples for serum glucose were collected into fluoride oxalate tubes and analyzed using an automated colorimetric method (Roche Diagnostics) (reference range, 3.89-6.38 mmol/L; CV intrabatch, 0.9%; interbatch, 1.8%). Serum insulin levels were determined using a solid-phase enzyme amplified sensitivity immunoassay (Linco Research Inc) (reference range, 2-200  $\mu\text{U/mL}$ ; CV intra-assay, 5.96%; interassay, 10.3%). HOMA scores were calculated as fasting glucose (mmol/L)  $\times$  fasting insulin ( $\mu\text{IU/mL}$ )/22.5.

### Isolation and culture of mature adipocytes and preadipocytes

In brief, 10 to 20 g (wet weight) of fresh AT was collected. Tissue was initially washed with  $1\times$  Hanks' balanced salt solution containing penicillin (100 U/mL) and streptomycin (100  $\mu\text{g/mL}$ ). Visible blood vessels and connective tissue

were removed, and the tissue was finely chopped. All AT was digested with the same batch of collagenase class 1 (2 mg/mL, Worthington Biochemical Corp) in  $1 \times$  Hanks' balanced salt solution (Gibco, Paisley, UK) for 20 minutes at  $37^{\circ}\text{C}$ .<sup>26</sup> The disrupted tissue was filtered through a double-layered cotton mesh, and preadipocytes and adipocytes were separated by centrifugation at 360g for 5 minutes. The upper layer of mature adipocytes was then removed from the collagenase-dispersed preparation, washed in phenol red-free medium Dulbecco modified Eagle medium (DMEM:F12) twice, and centrifuged at 360g for 2 minutes. The isolated adipocytes (1 mL, consisting of approximately 500,000 mature adipocytes) were maintained in flasks ( $25\text{ cm}^2$ ) of DMEM:F12 containing 15 mmol/L glucose, penicillin (100 U/mL) and streptomycin (100  $\mu\text{g/mL}$ ) for 48 hours.

### Protein extraction and quantification from adipose tissue and adipocytes

Proteins were extracted from whole AT using the Cambridge radioimmunoprecipitation assay protein extraction method.<sup>27</sup> AT (100 mg) was resuspended in 300  $\mu\text{L}$  radioimmunoprecipitation assay buffer and homogenized for 30 seconds (Fisher Scientific). The resulting mixture was then flash frozen, allowed to thaw at room temperature, and subsequently spun at 13,000 rpm for 30 minutes at  $4^{\circ}\text{C}$ . Infranant was collected and stored at  $-80^{\circ}\text{C}$ . The methodology for protein extraction from adipocytes and preadipocytes was identical to AT except that 500  $\mu\text{L}$  of the cell sample was mixed with 200  $\mu\text{L}$  of radioimmunoprecipitation assay buffer before flash freezing and spinning. After extraction, protein concentrations were determined using the Bio-Rad detergent-compatible protein assay kit.<sup>28</sup>

### RNA extraction from adipose tissue, adipocytes, and preadipocytes

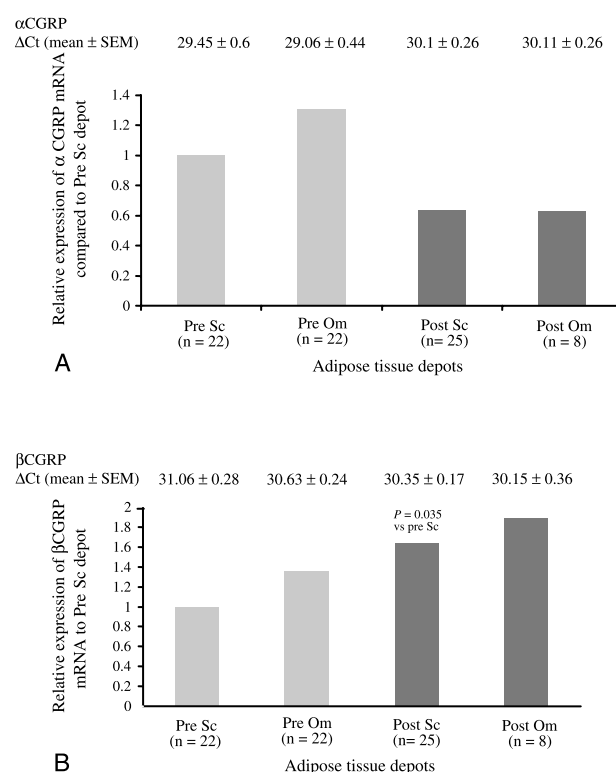
Total RNA was isolated from whole AT, adipocytes, and preadipocytes using a column-based method, according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit, Qiagen). After RNA extraction, a DNA digestion step was performed (DNase Kit, Sigma) to remove any contaminating genomic DNA. RNA from adipocytes and preadipocytes was also extracted in a similar manner. RNA (1  $\mu\text{g}$ ) from each sample was reverse-transcribed using RevertAid H minus M-MuLV reverse-transcriptase (Fermentas) and random hexamers, in accordance with the manufacturer's instructions.

### Real-time polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on the 7700 sequence detection system in 25  $\mu\text{L}$  volumes on 96-well plates in a reaction buffer containing Taqman universal PCR master mix (containing 1.25 U AmpliTaq Gold DNA polymerase, 1.25 U AmpErase UNG, 200  $\mu\text{mol}$  deoxyribonucleoside triphosphate with deoxyuridine triphosphate passive reference and optimized buffer component), 100 to 200 nmol Taqman probe, 900 nmol primers, and 15 to 30 ng cDNA. Previously detailed primer and probe sequences for the *CD45* gene were used to

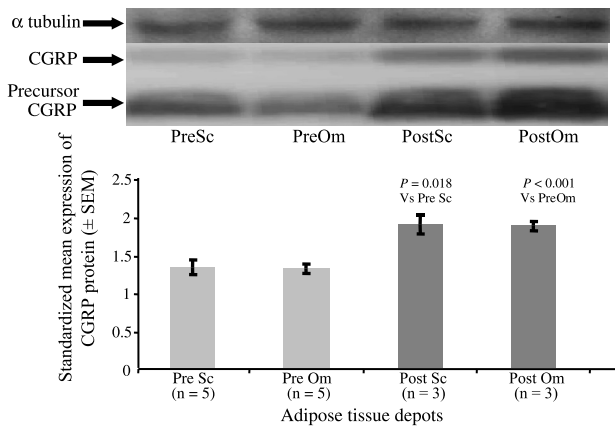
analyze *CD45* mRNA expression.<sup>27</sup> Previous studies have highlighted CGRP mRNA expression in macrophages.<sup>29,30</sup> Therefore, we assessed AT depots and isolated adipocytes, examining the impact of CGRP expression attributable to macrophages by using *CD45* as a marker for macrophages. Quantitative primer and probe sequences for *CD45* were forward primer 5' CGT AAT GGA AGT GCT GCA ATG T 3', reverse primer 5' CTG GGA GGC CTA CAC TTG ACA 3', and Taqman probe 5' ACA ACT AAA AGT GCT CCT CCA AGC CAG GTC T 3' (accession number NM\_080922).<sup>27</sup> The mRNA levels for  $\alpha\text{CGRP}$ ,  $\beta\text{CGRP}$ , ADM, RAMP1, RAMP2, and RAMP3 were analyzed using ABI predesigned gene expression assay-on-demand kits (Applied Biosystems, Warrington, UK). All reactions were multiplexed with the housekeeping gene *18S*, provided as a preoptimized control probe (Applied Biosystems), enabling data to be expressed in relation to an internal reference to allow for differences in reverse-transcription efficiency.

Reactions were as follows:  $50^{\circ}\text{C}$  for 2 minutes and  $95^{\circ}\text{C}$  for 10 minutes, and then 44 cycles at  $95^{\circ}\text{C}$  for 15 seconds and  $16^{\circ}\text{C}$  for 1 minute. The data were obtained as cycle threshold (Ct) values according to the manufacturer's guidelines (the cycle number at which the logarithmic PCR plot crosses a calculated threshold line) and used to determine  $\Delta\text{Ct}$  values ( $\Delta\text{Ct} = \text{Ct}$  of the gene of interest



**FIG. 1.** The mean relative fold difference in mRNA expression of  $\alpha\text{CGRP}$  (A) and  $\beta\text{CGRP}$  (B) in premenopausal omental (Pre Om), postmenopausal subcutaneous (Post Sc), and postmenopausal omental (Post Om) adipose tissue depots relative to the premenopausal subcutaneous (Pre Sc) adipose depot. The *18S* gene has been used as a preoptimized control probe (Applied Biosystems, Warrington, UK). CGRP, calcitonin gene-related peptide; Ct, cycle threshold.

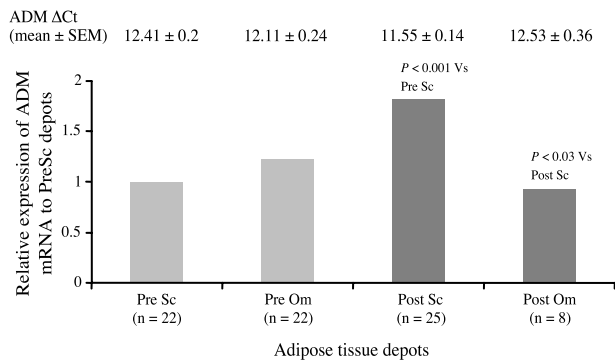




**FIG. 2.** The standardized mean expression of CGRP protein ( $\pm$  SEM) in human subcutaneous (Sc) and omental (Om) adipose tissue depots of premenopausal (Pre) and postmenopausal (Post) adipose tissue depots, by Western blot. Autoradiographs quantified by densitometry using a Gelbase/Gelblot program (UVP Ltd). Statistical analysis compared expression of CGRP in premenopausal depots versus postmenopausal depots. Representative Western blots for CGRP and the precursor protein are shown at top. The  $\alpha$  tubulin Western blot (uppermost) demonstrates equal loading of the protein. CGRP, calcitonin gene-related peptide.

minus Ct of the *18S* gene). A lower  $\Delta$ Ct value means the earlier appearance of the gene and thus higher expression of the gene. All measurements were carried out in triplicate for each sample. The individual  $\Delta$ Ct values were averaged for all samples for comparisons among participants and the depots of the same participants. Similarly, the  $\Delta$ Ct values for each group of AT biopsy samples (premenopausal Sc, premenopausal Om, postmenopausal Sc, postmenopausal Om) were averaged to calculate a group mean, SD, and SEM for each depot. Independent *t* tests were used to analyze the group means for each depot. The relative fold difference (as shown in Figs. 1-4) for all the mRNA expression was calculated using the premenopausal AbSc group as a reference, which was given an arbitrary value of 1. All the data were statistically analyzed at the  $\Delta$ Ct stage to avoid potential bias of averaging data that had been transformed through the equation  $2^{-\Delta\Delta\text{Ct}}$ .

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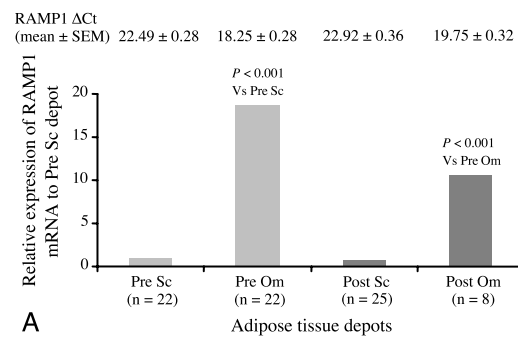


**FIG. 3.** The relative fold difference in mRNA expression of adrenomedullin (ADM) in premenopausal omental (Pre Om), postmenopausal subcutaneous (Post Sc), and postmenopausal omental (Post Om) adipose tissue depots relative to the premenopausal subcutaneous (Pre Sc) adipose depot. The *18S* gene has been used as a preoptimized control probe (Applera, Warrington, UK). Ct, cycle threshold.

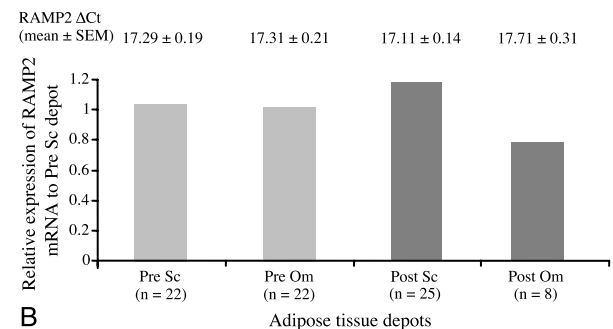
## Western blot

For Western blot analysis, 20  $\mu$ g of AT protein samples were loaded onto a 15% polyacrylamide gel. After gel electrophoresis and electroblotting, the membrane was incubated with a primary monoclonal mouse antibody for human CGRP (Biohit Diagnostics, Devon, UK). Detection of CGRP was achieved using a horseradish peroxidase-conjugated secondary antibody (Binding Site, Birmingham, UK) diluted 1:80,000 in phosphate-buffered saline-T (0.05%T). A chemiluminescent detection system (ECL Advanced, Amersham Pharmacia Biotech, Little Chalfont, UK) was used to visualize bands after x-ray exposure. In addition, 40  $\mu$ g of mature adipocytes and 40  $\mu$ g of preadipocytes were used to assess CGRP protein expression. Autoradiographs were quantified by densitometry using a Gelbase/Gelblot program (UVP Ltd).

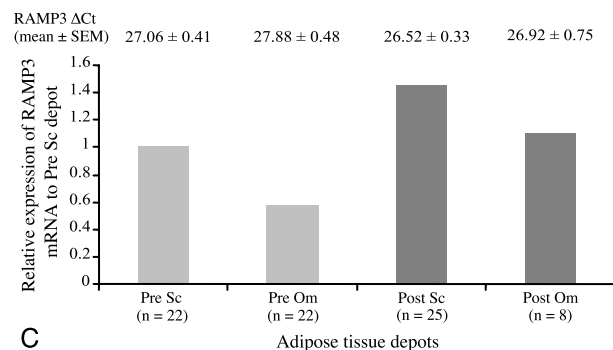
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A



B



C

**FIG. 4.** The relative fold difference in mRNA expression of RAMP1 (A), 2 (B), and 3 (C) in premenopausal omental (Pre Om), postmenopausal subcutaneous (Post Sc), and postmenopausal omental (Post Om) adipose tissue depots relative to the premenopausal subcutaneous (Pre Sc) adipose depot. The *18S* gene has been used as a preoptimized control probe (Applera, Warrington, UK). RAMP, receptor activity-modifying protein; Ct, cycle threshold.

### Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 12). Paired and unpaired Student *t* tests were used to analyze the expression of mRNA and proteins. Correlations for continuous variables affecting CGRP, ADM, and RAMP mRNA expression were determined by calculating Pearson correlation coefficients. The significance threshold was taken as  $P < 0.05$ . The data are presented as mean  $\pm$  SEM unless stated otherwise.

## RESULTS

### Baseline characteristics

As expected, the postmenopausal women were older (mean age: pre,  $43.3 \pm 1.51$  y; post,  $59.8 \pm 1.77$  y), had lower mean serum estradiol levels (pre,  $434.5 \pm 87.81$  pmol/L; post,  $43.3 \pm 6.95$  pmol/L), and higher mean serum FSH levels (pre,  $9.1 \pm 2.67$  IU/L; post,  $63.6 \pm 9.72$  IU/L). Mean systolic blood pressure was higher in the postmenopausal group ( $P = 0.015$ ); however, the two groups were comparable in mean diastolic blood pressure, BMI, waist circumference, waist-hip ratio, serum glucose, and insulin levels (Table 1).

### CGRP mRNA expression in adipose tissue, mature adipocytes, and preadipocytes

Both  $\alpha$ CGRP and  $\beta$ CGRP mRNA were expressed in AbSc and Om AT depots. The  $\alpha$ CGRP expression was similar in Sc and Om AT, but the expression was significantly lower in the Om depot from postmenopausal women compared with that from the premenopausal women (mean  $\pm$  SEM  $\Delta$ Ct: premenopausal Sc,  $29.45 \pm 0.6$ ; premenopausal Om,  $29.06 \pm 0.44$ ; postmenopausal Sc,  $30.1 \pm 0.26$ ; postmenopausal Om,  $30.11 \pm 0.26$ ) (Fig. 1A). The  $\beta$ CGRP showed higher expression in both Sc and Om adipose depots from the postmenopausal women than from the premenopausal women (mean  $\pm$  SEM  $\Delta$ Ct: premenopausal Sc,  $31.07 \pm 0.28$ ; premenopausal Om,  $30.63 \pm 0.24$ ; postmenopausal Sc,  $30.35 \pm 0.17$ ; postmenopausal Om,  $30.15 \pm 0.36$ ) (Fig. 1B). CGRP mRNA was also found to be expressed by mature adipocytes ( $n = 6$ ) and preadipocytes ( $n = 9$ ). However, this was not attributed to macrophage contamination, as CD45, the macrophage marker, remained undetected in preadipocytes and mature adipocytes.

**TABLE 1.** Population characteristics of premenopausal and postmenopausal groups

	Premenopausal (n = 22)	Postmenopausal (n = 25)	<i>P</i>
Age, y	43.32 (1.51)	59.80 (1.76)	<0.001
BMI, kg/m <sup>2</sup>	27.43 (1.03)	27.25 (0.79)	0.89
Waist circumference, cm	87.43 (2.47)	87.47 (3.03)	0.99
Waist-hip ratio	0.83 (0.01)	0.81 (0.02)	0.30
Mean systolic BP, mm Hg	125.33 (3.52)	145.56 (8.69)	0.05
Mean diastolic BP, mm Hg	75.05 (1.95)	83.00 (5.16)	0.086
Serum glucose, mmol/L	5.14 (0.20)	5.12 (0.21)	0.97
Serum insulin, $\mu$ U/mL	7.05 (1.34)	11.35 (3.65)	0.18

Values are mean (SEM). BP, blood pressure.

### CGRP protein expression in adipose tissue, mature adipocytes, and preadipocytes

Western blotting showed that CGRP was expressed in all AT depots (Fig. 2) as well as mature adipocytes and preadipocytes (data not shown). Sc and Om AT depots from the postmenopausal women showed higher expression of CGRP protein compared with the premenopausal women ( $P < 0.05$ ), although no difference in CGRP protein expression was observed between Sc and Om depots (Fig. 2).

### Expression of ADM mRNA in adipose tissue and mature adipocytes

ADM was highly expressed in all the AT depots (mean  $\pm$  SEM  $\Delta$ Ct: premenopausal Sc,  $12.41 \pm 0.2$ ; premenopausal Om,  $12.11 \pm 0.24$ ; postmenopausal Sc,  $11.55 \pm 0.14$ ; postmenopausal Om,  $12.53 \pm 0.36$ ). The postmenopausal Sc adipose depot showed a 1.8-fold increase in ADM expression compared with the premenopausal Sc tissue ( $P < 0.001$ ; Fig. 3). The ADM was also expressed in isolated adipocytes (data not shown).

### Expression of RAMPs (1, 2, 3) mRNA in adipose tissue and mature adipocytes

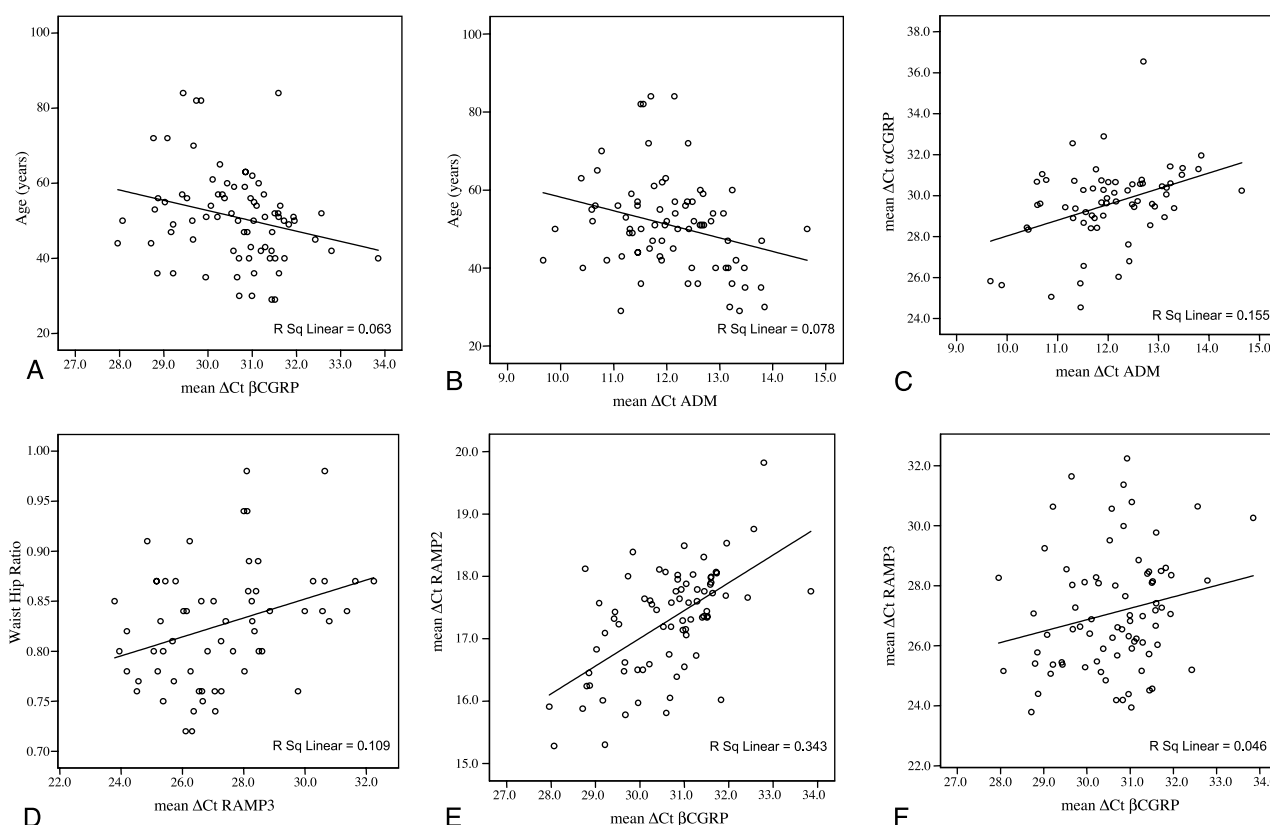
All three RAMPs were expressed in Sc and Om AT, with RAMP2 showing the highest expression overall. RAMP expression was not influenced by menopause status as no differential expression was observed (Fig. 4A-C). Only RAMP1 showed depot-specific gene expression, with the Om depots showing increased mRNA expression (pre Sc/pre Om, 1:6.6; pre Sc/post Om, 1:18). The RAMPs were also expressed in isolated adipocytes, with RAMP2 again showing greatest mRNA expression (mean  $\pm$  SEM  $\Delta$ Ct: RAMP1,  $22.66 \pm 0.94$ ; RAMP2,  $17.07 \pm 0.87$ ; RAMP3,  $29.08 \pm 1.55$ ).

### Effects of menstrual phase on CGRP, ADM, and RAMP mRNA expression

Among premenopausal women, the expression of  $\alpha$ CGRP mRNA was higher in women in follicular phase of the menstrual cycle compared with those in the luteal phase (mean  $\pm$  SEM  $\Delta$ Ct: follicular,  $28.11 \pm 0.54$ ; luteal,  $30.42 \pm 0.60$ ,  $P = 0.01$ ). No effect of menstrual phase was observed on mRNA expression of  $\beta$ CGRP, ADM, or any of the RAMPs.

### Effects of covariates on CGRP, ADM, and RAMP mRNA expression

ADM mRNA expression and  $\beta$ CGRP mRNA were positively correlated with age ( $\beta$ CGRP:  $r = -0.25$ ,  $P = 0.028$ ; ADM:  $r = -0.279$ ,  $P = 0.014$ ), suggesting a higher expression of these mRNA in older women.  $\alpha$ CGRP mRNA expression, however, did not show such a correlation, suggesting no influence of age on the expression of  $\alpha$ CGRP mRNA. ADM and  $\alpha$ CGRP were positively correlated with each other ( $r = 0.394$ ,  $P = 0.001$ ); however, there was no correlation of ADM and  $\alpha$ CGRP with any of the RAMPs.  $\beta$ CGRP mRNA expression was positively correlated with RAMP2 ( $r = 0.586$ ,  $P < 0.001$ ) and RAMP1 when controlled for age ( $r = 0.555$ ,  $P = 0.001$ ), the two RAMPs necessary for binding of  $\beta$ CGRP to the receptor indicating a simultaneous



**FIG. 5.** Bivariate correlations between mRNA expression (expressed as  $\Delta Ct$  values) of  $\beta$ CGRP with age (A), adrenomedullin (ADM) with age (B), ADM with  $\alpha$ CGRP (C), RAMP3 with waist-hip ratio (D),  $\beta$ CGRP with RAMP2 (E), and  $\beta$ CGRP with RAMP3 (F). CGRP, calcitonin gene-related peptide; Ct, cycle threshold; RAMP, receptor activity-modifying protein.

up-regulation of their mRNA expression. RAMP2 expression was positively correlated with RAMP3 mRNA levels ( $r = 0.346$ ,  $P = 0.002$ ). Furthermore, RAMP3 showed lower mRNA expression in women with increasing waist-hip ratio, ie, central adiposity ( $r = 0.33$ ,  $P = 0.009$ ) (Fig. 5A-F).

## DISCUSSION

CGRP is widely recognized as a neuropeptide released from sensory nerves in the central and peripheral nervous systems. The present study demonstrates that CGRP is actively expressed at the mRNA and protein level in human AT as well as in isolated mature adipocytes. In addition, these studies have explored the potential mechanism of CGRP action through ADM and RAMPs by examining whether alteration in menopausal status affects receptor expression, which may influence the role of CGRP as a vasodilator. Our findings have established that AT and, more specifically, isolated adipocytes act as a source of CGRP and ADM. Furthermore, AbSc AT taken from postmenopausal women has increased CGRP and ADM protein expression as compared with that from premenopausal women.

Because of the homogeneity of  $\alpha$ CGRP and  $\beta$ CGRP, it was not possible to discriminate between the two isoforms at the protein level. However, our mRNA findings suggest that the higher level of CGRP protein in the AT from postmenopausal women may arise from increased expression

of  $\beta$ CGRP, as the expression of  $\alpha$ CGRP mRNA was much lower in postmenopausal women compared with  $\beta$ CGRP. This is consistent with previous findings, which have highlighted  $\beta$ CGRP as the prevalent form in the enteric system.<sup>17</sup> Studies have also suggested that  $\alpha$ CGRP and  $\beta$ CGRP bind differently to the receptors and may have different responses to their antagonists. Whereas  $\alpha$ CGRP mainly binds to the CGRP<sub>1</sub> receptor, formed by the association of CRLR with RAMP1,  $\beta$ CGRP may also bind to the ADM receptor (ADM<sub>2</sub>) formed by the association of CRLR and RAMP3.<sup>31</sup> The linear correlation between  $\beta$ CGRP mRNA and RAMPs 1, 2, and 3 observed in our study indicates that, in AT,  $\beta$ CGRP may potentially be acting through the ADM receptors in addition to CGRP. The importance of this finding in vivo is not yet known.

CGRP is one of the many neuropeptides that have been studied to understand the complex pathogenesis of menopausal hot flashes. It is known that both neurogenic and humoral mechanisms are involved in the altered cutaneous thermoregulation, which is controlled by both the sympathetic and sensory nervous systems via release of peptides such as norepinephrine, neuropeptide Y, CGRP, and neurokinin A.<sup>32,33</sup> AT is a peripheral vascularized site that, by regulating the production of CGRP, may participate in regulating the cutaneous blood flow. Studies in rats have shown that neuropeptides derived from the sympathetic nerves located in white AT may influence lipid mobilization.<sup>34</sup>

Furthermore, CGRP was found to support recruitment and differentiation of periovarian brown adipocytes in cold acclimated rats, suggesting its active involvement in AT metabolism.<sup>35</sup> The presence of CGRP in AT not only supports the role of CGRP in maintaining body temperature, because of its potent vasodilatory ability, but also provides a possible mechanism by which AT may influence the severity of hot flushes in postmenopausal women. Local administration of CGRP-enhanced methacholine chloride induced sweating in human volunteers, indicating that the occurrence of sweats, after hot flushes, may be mediated through CGRP.<sup>36</sup> Visceral fat deposition is considered more pathogenic than Sc AT and has been shown to increase the relative risk of coronary heart disease-related deaths in postmenopausal women.<sup>37</sup> Visceral AT is metabolically more active than Sc AT because it is able to mobilize free fatty acids more readily.<sup>38</sup> Therefore, paired samples of Sc and Om AT were used in this study to investigate the differential expression of CGRP and ADM in relation to fat depot and to explore the mechanisms by which CGRP may influence cardiovascular risk. We found a significantly higher expression of RAMP1 in the Om fat tissue compared with the Sc tissue, which indicates a possible mechanism by which postmenopausal central adiposity may influence CGRP activity. Previous studies by Wimalawansa<sup>39</sup> suggest that age may potentially influence CGRP expression in certain tissues, although conflicting studies have shown no such relationship.<sup>40,41</sup> In the study by Schifter,<sup>40</sup> 232 healthy individuals (109 male, 123 female) between 18 to 79 years of age showed CGRP secretion to be unaffected by age. Similarly, age was not found to affect the tissue contents of CGRP in a study by Zaidi and colleagues.<sup>41</sup>

In a similar manner to CGRP, ADM also acts as a potent vasodilator and plays an important role in maintaining vascular homeostasis. AT has previously been reported to express ADM mRNA.<sup>42,43</sup> Our findings demonstrated that ADM was highly expressed by human AT and adipocytes, which is also influenced by menopause status. Overproduction of ADM has been found to be associated with the existence of cardiovascular disease, and increased circulatory levels may have a prognostic value in some cardiovascular diseases.<sup>20,21</sup> Our studies indicate that higher expression of ADM in AbSc AT, after menopause, may act as a contributing source to cardiovascular risk associated with menopause. Whether ADM is involved in vasodilation during hot flushes is, as yet, unknown, but intravenous administration of ADM has been reported to cause skin flushing, a phenomenon similar to CGRP.<sup>44</sup> Although ADM is mainly depicted as a circulatory peptide, local paracrine effects in AT may represent an important site to maintain normal microvasculature homeostasis. As such, increased ADM mRNA expression in AT from postmenopausal women indicates its possible involvement in the pathogenesis of hot flushes along with CGRP.

It should be noted that in the study design, it was important to ascertain BMI-matched pre- and postmeno-

pausal women to ensure that the effects we observed between the two cohorts were due to menopause status and not adiposity. As such, this has limited the ability to examine the role of adiposity since the cohorts collected span a narrow BMI range. Consequently no significant correlations between  $\alpha$ CGRP or  $\beta$ CGRP mRNA expression with BMI were noted; subsequent studies should examine this in further detail. In addition, because of our exclusion criteria, this study was unable to examine patients who had previously detailed severe vasomotor symptoms, such as those patients on HT. These patients were excluded because HT would influence AT metabolism and therefore may have influenced CGRP expression. However, future studies should explore the effects of estrogen directly on CGRP expression in AT as well as the effects of increased BMI on CGRP expression.

## CONCLUSION

In conclusion, our studies demonstrated that AT may represent an important site for the production and local action of CGRP and ADM as important vasodilators. In addition, the mRNA expression of  $\beta$ CGRP with RAMP2 and RAMP3 positively correlated in human AT, suggesting the possibility that  $\beta$ CGRP may act via the ADM receptors. Taken together, these findings highlight the potential of AT as a site of vasodilatory action, which our studies have shown to be exacerbated by menopause status, with both ADM and CGRP potentially contributing to menopausal hot flushes as well as being associated with increased microvascular disease associated with age.

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